IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of	: Naoyuki MORIYA et al.
Serial No.	: 10/531,463
PCT Filed	: March 4, 2004
For	: COMPOSITION CONTAINING BETA-GLUCAN AND CONSTIPATION-RELIEVING DRUG, IMMUNOPOTENTIATOR AND SKIN MOISTENING AGENT USING THE COMPOSITION
Art Unit	: 1623
Examiner	: BLAND, LAYLA D

DECLARATION UNDER 37 CFR 1.132

HONORABLE COMMISSIONER OF PATENTS AND TRADEMARKS

WASHINGTON, D.C. 20231

SIR;

Now comes Koji KUBOTA who deposes and says that:

1. I am an employee of AUREO CO., LTD..

2. I graduated from Tokyo University of Fisheries in 1962 , and received Ph.D. degree from Kyoto University in 1990 , and has been employed by AUREO CO. LTD. since 2003 .

3. Under my supervision and control, the following experiments were carried out:

EXPERIMENTS

(1) β-glucan containing composition

An *Aureobasidium pullulans* cultured composition was obtained as in Example 1 of the present application 10/531,463. On the other hand, as comparison, a commercially available β-glucan containing composition "senseiro"(trade name, distributed by KYOWA ENGENEERING CO.) was employed. The "senseiro" contains extracts from fruiting body of *Agaricus blazei Murill*. This "senseiro" was the one used in the cited prior art Wagon et al. (JP 2003-040875, see [0036] in the Japanese language publication).

With respect to the above-mentioned composition, β-glucan concentration was measured as described in Example 1 of the present application 10/531,463. Specifically, the composition is subjected to enzyme treatment with amylase, amyloglucosidase, protease, etc., and proteins and α-glucan such as pullulans are removed, followed by ethanol precipitation. Furthermore, filtration is performed using a glass filter, to thereby yield a resultant of substances with high-molecular weight. In such step, the resultant of substances with high-molecular weight is sufficiently washed with 80% ethanol in order to remove substances with low-molecular weight including monosaccharides. The washed high-molecular sample is further washed with acetone, and sulfuric acid is added thereto for hydrolysis. After hydrolysis, neutralization is performed, and the filtered solution is collected. Quantification of glucose is performed by the glucose oxidase method, and the value calculated based

on the following mathematical expression 1 is defined as the glucan amount.

: β-glucan (g/100 g) = glucose (g/100 g) × 0.9

The result was that *Aureobasidium* cultured composition contained 30 % by mass of β-glucan (β-1,3-1,6-glucan) with respect to the solid content, while the comparison composition "senseiro" contained 2.79 % by mass of β-glucan with respect to the solid content.

(2) Lactic acid bacterium cells of *Enterococcus faecalis* killed by heat treatment

Lactic acid bacterium cells of *Enterococcus faecalis* killed by he at treatment was obtained as in Example 1 of the present application 10/531,463.

Specifically, an appropriate amount of pre-culture obtained by culturing *Enterococcus faecalis* (IFO 16803) in Rogosa medium at 37°C for 24 hours was inoculated in a liquid medium containing 4% of yeast extracts, 3% of polypeptone, and 10% of lactose, and neutralization culture was performed at 37°C for 22 to 24 hours while the pH value of the medium was controlled to pH 6.8 to 7.0 with an aqueous solution of sodium hydroxide using a pH stat.

After completion of the culture, the bacterium cells were separated and collected using a continuous centrifuge. Thereafter, water was added thereto for diluting to the former liquid amount, and the bacterium cells were separated and collected again using the continuous centrifuge. The

operations were repeated four times in all for washing the bacterium cells. Subsequently, the washed bacterium cells were suspended in an appropriate amount of water, and the mixture was sterilized at 100°C for 30 minutes. Then, the bacterium cells were dried using a spray drier, to thereby prepare powder of heat-sterilized bacterium cells (5×10^{12} cfu/g).

(3) Viscosity of the β-glucan containing composition and dispersion of the cells when mixed with the composition

With 100 g of the *Aureobasidium pullulans* cultured composition (a solid content of 0.70 % by mass), Lactic acid bacterium cells of *Enterococcus faecalis* killed by heat treatment was suspended so as to be a mixed composition with a concentration of 2.9×10^{10} cfu/g of the cells. Hereinafter this mixed composition is referred to as 'mixed composition 1'.

With 100 g of the β-glucan containing composition "senseiro"(trade name, distributed by KYOWA ENGENEERING CO.) (a solid content of 1.08 % by mass), Lactic acid bacterium cells of *Enterococcus faecalis* killed by heat treatment was suspended so as to be a mixed composition with a concentration of 2.9×10^{10} cfu/g of the cells. Hereinafter this mixed composition is referred to as 'mixed composition 2'.

Further, by dehydration of the β-glucan containing composition "senseiro" in 'mixed composition 2', the β-glucan content in the mixed composition was adjusted to a concentration as same as that in 'mixed composition 1'. Hereinafter this mixed composition is referred to as 'mixed composition 3'.

The viscosity of the mixed composition was measured using a viscometer (trade name "TVC-5 type" distributed by TOKISANNGYOU K.K.) and a viscometer (trade name "VM-10A" distributed by CBC MATERIALS K.K.). The result in a measurement using a viscometer "TVC-5 type" was 203 mPas with 'mixed composition 1' while it was 4.6 mPas with 'mixed composition 2'. The result in a measurement using a viscometer "VM-10A" was 7 mPas with 'mixed composition 1' while it was 2 mPas with 'mixed composition 2'. These results showed that the viscosity of 'mixed composition 1' was higher than that of 'mixed composition 2'

Further, with 'mixed composition 3' in which the β-glucan content was adjusted to a concentration as same as that in 'mixed composition 1', the result in a measurement using a viscometer "VM-10A" was 2 mPas comparable with 'mixed composition 2', showing that the viscosity was not correlated with the solid content in the mixed composition. Therefore, it was suggested that this feature of 'mixed composition 1' was attributed to the source of β-glucan, which was *Aureobasidium pullulans*.

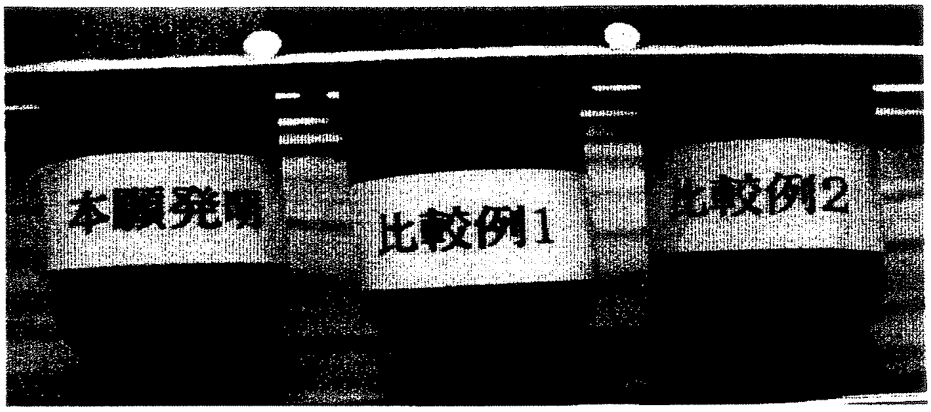
The dispersion of the *Enterococcus faecalis* cells when mixed with the composition was observed by eye. In 'mixed composition 2' and 'mixed composition 3', precipitates of the cells occurred in 5 min after suspension of cells in the β-glucan containing composition, while precipitates of the cells were not observed in 'mixed composition 1'. Fig. 1 attached below shows the pictures at 3 hours after suspension of cells. Notably, over 1-year period of the time, the dispersion of the cells in 'mixed composition 1' had been stable.

In order to detect the *Enterococcus faecalis* cells present in an upper portion of the respective 'mixed composition' stayed in a test tube, a cohesion assay was performed using specific polyclonal antibody against the *Enterococcus faecalis* cells. In this assay, if cells are not present, latex beads in the assay solution are precipitated because of the gravity. If cells are present, the latex beads are dispersed because of the aggregation with the cells, which can be determined by eye.

The result is shown in Fig. 2 attached below. From the upper portion of 'mixed composition 1', the *Enterococcus faecalis* cells were detected. On the other hand, they were detected neither from the upper portion of 'mixed composition 2' nor that of 'mixed composition 3'.

[Figure 1]

mixed composition 1 mixed composition 2 mixed composition 3

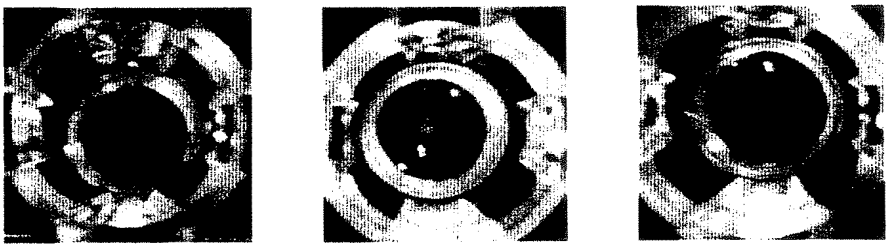


Uniformly dispersed Precipitated Precipitated

[Figure 2]

Presence of Lactic acid bacterium cells
at the upper portion of the composition

mixed composition 1 mixed composition 2 mixed composition 3



Detected Not detected Not detected

SUMMARY

The above-mentioned results are summarized in Table 1.

[Table 1]

	'mixed composition 1'	'mixed composition 2'	'mixed composition 3'
Source of β-glucan	*Aureobasidium pullulans*	*Agaricus blazei Murill*	*Agaricus blazei Murill*
Lactic acid bacterium	*Enterococcus faecalis*	*Enterococcus faecalis*	*Enterococcus faecalis*
β-glucan with respect to the solid content (mass %)	30.00	2.79	2.79
Concentration of β-glucan (mg/ml)	2.1	0.3	2.1
Lactic acid bacterium cells (cfu/ml)	2.9×10^{10}	2.9×10^{10}	2.9×10^{10}
Viscosity measured using "VM-10A" (m.Pas)	7	2	2
Viscosity measured using "TVC-5 type" (m.Pas)	203	4.6	Not determined.
Dispersion of Lactic acid bacterium cells	Uniformly dispersed	Precipitated	Precipitated

4. Consideration

The β-glucan (β-1,3-1,6-glucan) contained in the *Aureobasidium pullulans* cultured composition includes different characteristics from the β-glucan obtained from fruit body of *Agaricus blazei Murill*, which leads to difference on

viscosity of the β-glucan containing composition and to good properties of dispersion of cells of Lactic acid bacterium *Enterococcus faecalis* in the mixed composition.

The results suggest that, because β-glucan is secreted from cells of *Aureobasidium pullulans* into the culture medium when cultivated, a certain natural three dimensional structure of the β-glucan (β-1,3-1,6-glucan) could not be destructed to remain. On the other side, an extraction step by hot water or the like is required to extract β-glucan from fruit body of *Agaricus blazei Murill*, which could lead to destruction of the natural state of β-glucan.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements are the like so made are punishable by fine or imprisonment, or both, under Section 1001, of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Dec. 5, 2007

Koji Kubota

Koji KUBOTA